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# Mechanism of Androgen Receptor Corepression by CKβBP2/CRIF1, a Multifunctional Transcription Factor Coregulator Expressed in Prostate Cancer

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## Abstract

The transcription factor coregulator Casein kinase IIβbinding protein 2 or CR6-interacting factor 1 (CKβBP2/CRIF1) binds the androgen receptor (AR) in prostate cancer cells and in response to dihydrotestosterone localizes with AR on the prostate-specific antigen gene enhancer, but does not bind DNA suggesting CKβBP2/CRIF1 localization in chromatin is determined by AR. In this study we show also that CKβBP2/CRIF1 inhibits wild-type AR and AR N-terminal transcriptional activity, binds to the AR C-terminal region, inhibits interaction of the AR N- and C-terminal domains (N/C interaction) and competes with p160 coactivator binding to the AR C-terminal domain, suggesting CKβBP2/CRIF1 interferes with AR activation functions 1 and 2. CKβBP2/CRIF1 is expressed mainly in stromal cells of benign prostatic hyperplasia and in stroma and epithelium of prostate cancer. CKβBP2/CRIF1 protein is increased in epithelium of androgen-dependent prostate cancer compared to benign prostatic hyperplasia and decreased slightly in castration recurrent epithelium compared to androgen-dependent prostate cancer. The multifunctional CKβBP2/CRIF1 is a STAT3 interacting protein and reported to be a coactivator of STAT3. CKβBP2/CRIF1 is expressed with STAT3 in prostate cancer where STAT3 may help to offset the AR repressor effect of CKβBP2/CRIF1 and allow AR regulation of prostate cancer growth.

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transcription coactivators and corepressors; protein kinase CK2 binding protein 2; CR6 interacting
factor-1; signal transducer and activator of transcription 3

## 1. Introduction

The androgen receptor (AR) has an essential role in human male reproductive tract
development and function as evidenced by AR gene mutations that result in the androgen
insensitivity syndrome (Quigley et al., 1995). AR is a DNA binding transcription factor that
directs the cellular actions of androgens by acting as a docking platform for transcription
coregulators. Epithelium and stroma of human prostate are targets of androgen action and
depend on AR and its natural ligand, dihydrotestosterone (DHT) for growth and male sexual
development. Similarly, neoplastic growth of the prostate is AR dependent and regresses
initially in response to androgen deprivation therapy. Evidence indicates that AR has a
central role in the eventual relapse of prostate cancer during androgen deprivation therapy
(referred to as castration-recurrent or castration-resistant growth) (Grossman et al., 2001;
Gregory et al., 2001a, 2001b; Ponguta et al., 2008; Mohler et al., 2004; Titus et al., 2005;
Yuan and Balk, 2009; Agoulnik and Weigel, 2006; Heemers and Tindall, 2007).
Transcriptional functions of AR involve the concerted actions of coactivators and
corepressors (Heemers and Tindall, 2007; Wang et al., 2005; Burd et al., 2006) that regulate
chromatin modifying complexes and gene transcription (reviewed in Perissi et al., 2010;
Battaglia et al., 2010)

CKβBP2/CRIF1 cDNA was isolated originally in p53 stably transformed colon cancer cell
lines (Horikoshi et al., 1999) and later identified as a protein that interacts with the β subunit
of protein kinase CK2 (Ahn et al., 2001), but its influence on the function of protein kinase
CK2 remains unknown. CKβBP2/CRIF1 was identified also as one of a number of
papilloma virus L2 interacting nuclear proteins (Gornemann et al., 2002). Indication of a
function for CKβBP2/CRIF1 was revealed when Chung et al. (2003) reported CKβBP2/
CRIF1 is a GADD45γ binding protein. GADD45α, β and γ are DNA damage inducible
proteins (Carrier et al., 1999) that inhibit the cell cycle. CKβBP2/CRIF1 interacted with
GADD45γ and had a similar function with respect to cell cycle inhibition (Chung et al.,
2003).

Here we report a mechanism for the AR corepressor activity of CKβBP2/CRIF1 (Su JH et
al. 2008) and identify a small fragment of CKβBP2/CRIF1 that inhibits the transcriptional
activity of AR. CKβBP2/CRIF1 interacted with the C- and N-terminal regions of AR,
disrupted the AR N/C interaction and competed with TIF2 binding to the AR C-terminus.
CKβBP2/CRIF1 also inhibited the constitutive activity of an AR N-terminal-DNA binding
domain fragment. CKβBP2/CRIF1 protein expression was analyzed in prostate cancer where
AR has a major role in neoplastic growth and progression. In benign prostatic hyperplasia,
CKβBP2/CRIF1 protein was expressed mainly in fibromuscular stromal cells. In androgen-
dependent prostate cancer, CKβBP2/CRIF1 protein was increased in epithelial cells
compared to benign prostatic hyperplasia. However, it was reduced in epithelial cells of
castration-recurrent compared to androgen-dependent prostate cancer.

STAT3 is known to promote prostate cancer progression. In prostate cancer tissue
microarrays, we show that CKβBP2/CRIF1 and STAT3 proteins are present in the same
cellular locations. CKβBP2/CRIF1 is a STAT3 coactivator (Kwon et al., 2008). CKβBP2/

CRIF1 binding and coactivation of STAT3 may offset the AR repressor effect of CKβBP2/CRIF1 and help maintain a selective advantage for prostate cancer cell growth.

## 2. Materials And Methods

### 2.1 Yeast two hybrid screening

The AR vector pBDGAL4CAM-481–919 that expresses the carboxyl-terminal portion of the human AR N-terminal, DNA and ligand-binding domains was used as bait in yeast two-hybrid screening of a human epithelial cervical carcinoma HeLa cell cDNA library in GAD-GH (Clontech Laboratories, Inc., Palo Alto, CA) (gift of Yue Xiong, University of North Carolina at Chapel Hill). Yeast strain Hf7c co-transformed with HeLa cell cDNAs was plated on synthetic medium without Leu, Trp and His containing 5 mM 3-amino-1,2,4-triazole and 1 μM DHT. After 5 to 7 days in culture, colonies were selected for βgalactosidase assay. cDNAs from βgalactosidase positive yeast colonies were rescued using standard procedures. Liquid βgalactosidase assays were performed as described for the Clontech Matchmaker system (Clontech protocol PT3247-1).

### 2.2 Plasmids

The coding region for CKβBP2/CRIF1 was excised from pGAD-GH using EcoRI and XhoI and cloned into the same restriction sites in pSG5 (Stratagene) with a FLAG tag added to make pSGFLAG-CKβBP2/CRIF1-7-222. pSG5 (pSG) was chosen for these studies because its SV40 promoter was not affected by pSGFLAG-CKβBP2/CRIF1, whereas the activity of a CMV promoter was increased by pSGFLAG-CKβBP2/CRIF1. The cytomegalovirus-luciferase reporter vector was provided by William M. Wood and Arthur Gutierrez-Hartmann and SV40-luciferase was from Stratagene. pSGFLAG-CKβBP2/CRIF1-7-222 was digested with PstI, gel purified and the 6 kb fragment self-ligated to create pSGFLAG-CKβBP2/CRIF1-7-157. The small PstI fragment was treated with T4 DNA polymerase and cloned into pSGFLAG EcoRV to create pSGFLAG-CKβBP2/CRIF1-158-206. Full-length CKβBP2/CRIF1 was obtained from ATCC (catalog #6709344). pOBT7 containing full-length CKβBP2/CRIF1 was digested with EcoRI, treated with Klenow and digested with XhoI. The full-length fragment was cloned into pSGFLAG treated with BamHI, filled-in with Klenow followed by XhoI digestion to make pSGFLAG-CKβBP2/CRIF1-2-222. The full-length clone does not contain the codon T187C missense mutation we found in the pSGFLAG-CKβBP2/CRIF1-7-222 isolated by yeast two-hybrid screening. Human AR expression vectors GAL-AR-624-919, VP-AR-1-660 and VP-TIF2.1 (TIF2-624-1287) were described (Tan et al., 2000; He et al., 1999). The prostate-specific antigen (PSA) luciferase (Luc) reporter vector PSA-Enh-Luc provided by Michael Carey, University of California at Los Angeles, contains the PSA upstream enhancer region (Huang et al., 1999). MMTV-Luc reporter gene was provided by Stanley M. Hollenberg and Ron M. Evans (Salk Institute).

### 2.3 Transient cotransfection assay

Cotransfection assays were performed in triplicate (Tan et al., 2000; He et al., 1999; Bai et al., 2005, 2008). In brief, African green monkey kidney CV1 cells were maintained in 10% fetal bovine serum containing high glucose and antibiotics. Cells ($4.2 \times 10^5$) were plated in 6 cm ($28 \text{ cm}^2$) culture dishes. On the following day when 70-80% confluent, cells were transfected with MMTV-Luc (0.5 μg) and various amounts of expression vectors using the $CaPO_4$ method. After 15% glycerol shock for 4 min, cells were incubated in Dulbecco's modified Eagle's medium-H (DMEM-H) without phenol red and serum in the absence and presence of DHT. The next day media were removed and replaced with fresh media of the same composition. Incubation was continued for 20 h. Cells were harvested in lysis buffer and luciferase activity was measured in an automated multiwell luminometer. Results are

representative of at least three individual experiments performed in triplicate. Error bars represent ± SEM.

CWR-R1 cells were maintained in Richter's improved minimal essential medium (Irvine Scientific, Santa Ana, CA) supplemented with 10 mM nicotinamide, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 100 units/ml penicillin, 100 μg/ml streptomycin and 2% fetal bovine serum. Cells were plated in 6 well plates ($3.5 \times 10^6$ cells per well). Two hybrid interaction assays were performed in HeLa cells (Bai et al., 2005, 2008; Gregory et al., 2004) maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Gemini or Hyclone), penicillin, streptomycin and 2 mM L-glutamine. HeLa cells ($5 \times 10^4$ cells/well in 12 well plates) were transfected with 0.1 μg of 5xGAL4Luc3, 50 ng of VP-AR-1-660 or VP-TIF2.1-624-1287 and 50 ng GAL-AR-624-919. DNA was added to 43 μl of serum-free medium and 0.6 μl of FuGENE-6 reagent per well. After 15 min incubation, 40 μl of FuGENE DNA mixture was added to each well containing 1 ml medium. The next day, cells were washed with phosphate buffered saline (PBS) and 1 ml/well serum-free medium lacking phenol red and containing the indicated steroids was added and incubated overnight at 37°C. The next day cells were washed with PBS and assayed for luciferase activity after harvesting in 0.25 ml lysis buffer.

## 2.4 Glutathione S-transferase (GST) pull-down assays

GST binding assays were performed as described (Tan et al., 2000). In brief, pSGFLAG-CKβBP2/CRIF1 was incubated with [$^{35}$S]methionine and rabbit reticulocyte lysate using the TnT T7 Quick-coupled Transcription/Translation System kit (Promega). Labeled protein was distributed equally among incubations with GST-glutathione-Sepharose control and recombinant GST-fusion proteins bound to glutathione-Sepharose. After incubation and washes in the presence of 0.1 nM DHT, labeled protein was eluted by boiling in SDS buffer and separated by SDS-PAGE. Gels were dried and exposed to Kodak x-ray film.

## 2.5 Coimmunoprecipitation

COS cells cultured in Dulbecco's modified Eagle's medium-H in 10 cm dishes were transfected with pSGFLAG-CKβBP2/CRIF1-7-222, -7-157 or -158-206 in combination with pSG-AR using the DEAE-dextran method (Tan et al., 2000). Two days after transfection, cells were harvested in PBS and lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% NP40, 50 mM NaF with the addition of 0.8 mM DTT, 1 mM PMSF and a protease inhibitor cocktail pellet (Roche). 25 μl anti-FLAG M2 agarose gel (Sigma) was added and incubated overnight at 4°C. Beads were washed several times with lysis buffer and bound proteins eluted in SDS sample buffer, separated in SDS-PAGE and western blotted using FLAG M2 or AR32 antibody (Quarmby et al., 1990). The FLAG-STAT3 expression vector was pRC/CMV-STAT3 from Addgene.

## 2.6 Immunofluorescence microscopy

COS cells seeded on cover slips in 12-well plates ($1.25 \times 105$ cells/well) were transfected using the Effectene method with 0.1 μg pCMV-AR, pSGFLAG-CKβBP2/CRIF1 or pSGFLAG in the presence and absence of DHT as described (Bai et al., 2005). Cells were fixed in 3% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 5 min at 4°C. After rinsing with PBS 3 times, 0.5% bovine serum albumin in PBS was added and cells incubated 30 min at room temperature. Cells were incubated with primary antibodies anti-AR Abcam ab3510 (1:250 dilution) and/or anti-FLAG (1:1000 dilution) for 1 h at room temperature. After washing with PBS, a 1:50 dilution of secondary fluorescent antibody was added and incubated for 30 min. Microscopy was performed using an Olympus BX60 microscope with 40× objective, magnification 400×. Rabbit anti-AR ab3510 was from Abcam, Cambridge, MA. Mouse anti-FLAG

antibody was from Sigma. Fluorescein (FITC)-conjugated AffiniPure donkey anti-rabbit IgG and rhodamine (TRITC)-conjugated AffiniPure donkey anti-mouse IgG were from Jackson ImmunoResearch, West Grove PA.

## 2.7 Chromatin immunoprecipitation

CWR-R1 human prostate cancer cells ($10^7$/10 cm dish) were serum starved for 24 h. The next day, cells were changed to serum-free medium containing 10 nM methyltrienolone (R1881), incubated for various times and cross-linked using 1% formaldehyde for 10 min at room temperature followed by 5 min with 0.125 M glycine at room temperature. Cells were washed and harvested in PBS. Cell pellets were resuspended in 10 mM Hepes, pH 7.9, 0.5% Triton X-100, 1.5 mM $MgCl_2$, 10 mM KCl, 0.6 mM phenylmethylsulfonyl fluoride, 2.5 nM pepstatin A, 30 nM leupeptin and 0.5 mM DTT. After a 10 min incubation at 4°C, cell pellets were resuspended in 20 mM Hepes, pH 7.9, 25% glycerol, 0.5% Triton X-100, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA with the same amount of proteinase inhibitors. Following 20 min incubation at 4°C, cells were sonicated at 21% power 40 times, 0.5 sec each time and the procedure repeated to obtain 100-1000-bp DNA fragments. Following 10 min centrifugation, clarified lysates were diluted 1:1 with 1% Triton X-100, 2 mM EDTA, 50 mM NaCl, 20 mM Tris-HCl, pH 8.1 containing the same amounts of protease inhibitors. Samples were precleared for 1 h at 4°C with 20 µl of protein-A-agarose (Sigma). Immunoprecipitation was performed overnight at 4°C with the addition of 5 µg normal rabbit serum IgG (Santa Cruz Biotechnology sc-2027), rabbit anti-AR H-280 (Santa Cruz Biotechnology sc-13062) or CKβBP2/CRIF1 antibody. Samples were incubated with 20 µl of protein-A agarose for 3 h and protein-A agarose pellets washed sequentially with 1 ml each of low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1 and 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1 and 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1 and 1% sodium deoxycholate) and 2× 1 ml TE buffer (10 mM Tris-HCl, pH 8.1, 1 mM EDTA). Immunoprecipitated DNA was eluted in 50 µl of 1% SDS, 0.1 M $NaHCO_3$ 3 times at 25°C for 15 min each. The resulting DNA was de-crosslinked by adding 6 µ of 5 M NaCl, incubated at 65°C overnight, followed by 45°C incubation with the addition of 2.4 µl of 0.5 M EDTA, 4.8 µl of 2 M Tris-HCl, pH 6.5, 2 µl of 10 mg/ml proteinase K and 2 µl of 10 mg/ml RNase A for 1 h. DNA was purified and PCR performed in 10 µl reactions using SYBR green and PSA gene upstream enhancer primers 5′-GGGACAACTTGCAAACCTG-3′ and 5′-GTATCTGTGTGTCTTCTGAGC-3′ at 95°C for 20 min, 40 cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 45 sec to amplify a 285 bp fragment from the -4234 to -3950 5′-flanking region.

## 2.8 DNA binding assay

COS cells ($1.8 \times 10^6$/10 cm dish) transfected with 5 µg empty vector control (pSG-FLAG), pSGFLAG-AR or CKβBP2/CRIF1 in the presence of 10 nM DHT were harvested in PBS and lysed with 250 µl buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.1% SDS, 50 mM NaF, 2 mM sodium vanadate, 2 mM EDTA, 50 mM Tris-HCl, pH 7.6 plus 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and complete protease inhibitor cocktail (Roche Applied Science). The same PSA upstream enhancer DNA amplified in the ChIP assay (5 µl of 10 µlM stock) was added to 50 µl COS cell protein extract and 90 µl DNA binding buffer (10 mM Tris, pH 7.5, 25 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) with 1 mM $ZnSO_4$, incubated for 3 h at 4°C, 25 µ of M2-FLAG antibody-agarose beads added and incubation continued overnight at 4°C. The next day, agarose beads were washed 3 times with 1 ml DNA binding buffer with $ZnSO_4$. DNA binding buffer (100 µl) containing RNase and proteinase K was added to the beads and incubated at 65°C for 1 h. The mixture was phenol extracted, ethanol precipitated, dissolved in 10 µl of $H_2O$ and separated in a 1% agarose gel. The area of gel corresponding to the PSA

upstream enhancer DNA was cut out, DNA was eluted and used for RT-PCR. The amount of RT-PCR amplified DNA was calculated based on a PSA standard curve using genomic DNA. Fold binding was calculated against empty vector control (pSGFLAG) set to 1.

## 2.9 siRNA transfection

CKβBP2/CRIF1 siRNAs were obtained from Dharmacon, Chicago, IL (5′-3′ sense sequences siRNA1 [197]CUAAGCAGUUCGCGCGUUAUU, siRNA2 [264]GAGCUGGAGGCCGAAGAACUU). siRNAs were transfected into COS cells along with pSGFLAG-CKβBP2/CRIF1 or pSGFLAG empty vector using Lipofectamine 2000 (Invitrogen) according to the manufacture's instructions. COS cells were plated in 6 cm dishes at $3 \times 10^5$/dish (50-60% confluent) and cultured in DMEM-H without the addition of antibiotics. Lipofectamine diluted 1:10 in Opti-MEM medium was incubated at room temperature for 5 min and added to Eppendorf tubes containing siRNA and vector DNA, incubated an additional 20 min at room temperature and mixed before adding to the cells. COS cells were incubated for 48 h at 37°C and harvested in PBS. CV1 cells plated in 6 cm dishes at $4.5 \times 10^5$ cells/dish and cultured in DMEM-H. CWR-R1 cells in 6 well dishes at $4 \times 10^5$ cells/well were cultured in Richter's medium transfected with Lipofectamine as above.

## 2.10 Immunoblots

Antibodies were raised in rabbits with CKβBP2/CRIF1 peptide [144]CEKAQADKERRARL-QA[158] (Pocono Rabbit Farm & Laboratory, Inc. PA) and purified using peptide antigen affinity chromatography. For antibody assay, western blots were performed using protein extracts from COS cells transfected with pSGFLAG or pSGFLAG-CKβBP2/CRIF1.

The small repressor CKβBP2/CRIF1 protein fragment amino acids158-206 was expressed in COS cells and identified by chromatography on a 4-20% gradient gel.

## 2.11 Immunohistochemistry

Surgical specimens of prostate were collected according to NIH guidelines with approval by the IRB of Roswell Park Cancer Institute and the University of North Carolina School of Medicine at Chapel Hill. BPH cores were taken from radical prostatectomy specimens at sites away from the cancer. Tissue microarray (Titus et al., 2005) was performed as follows: formalin-fixed, paraffin-embedded tissue microarray sections were incubated at 70°C for 15 min, deparaffinized in toluene and rehydrated through graded alcohols to phosphate buffered saline. Endogenous peroxidase was blocked using 5% $H_2O_2$ in 83% methanol for 30 min at room temperature. For antigen retrieval, slides were placed in 0.01 M sodium citrate, 0.01 M critic acid, pH 6.0 for 15 min in a microwave on high setting followed by 15 min cool down incubation in the citrate solution (Balaton et al., 1993). Sections were blocked with 1.5% normal goat serum (for anti-CKβBP2/CRIF1) or rabbit serum (for anti-STAT3) for 20 min and incubated overnight at 4°C with anti-CKβBP2/CRIF1 (1:100 dilution) or anti-STAT3 ab30646 antibodies (1:100 dilution, Abcam). Slides were blocked in normal serum for 10 min and incubated at room temperature for 30 min with biotinylated anti-rabbit IgG (1:200 dilution) (Vector Labs, Burlingame, CA) followed by a 30 min incubation at room temperature with the Elite Avidin DH-biotinylated horseradish peroxidase H complex (1:50 dilution) (Vector Labs). Sections were incubated for 10 min with 3, 3′diamino-benzidine (DAB Peroxidase Substrate Kit SK-4100, Vector Labs) and counterstained with Gill's hematoxylin (Protocol Hematoxylin Gill-3X, Fisher Scientific). APERIO Imagescope software was used to capture and store the images. Defective cores with tissue damage were excluded from scoring for CKβBP2/CRIF1 and STAT3 staining . The analysis shown in Fig. 8 included 25 cores each of BPH, androgen-dependent and castration-recurrent prostate cancer.

### 2.11 Realtime RT-PCR

RNA was extracted using Trizol (Qiagen) according to manufacturer's recommendations. RNA (2 µg) was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen). RT-PCR using QuauntiTech SYBR Green (Qiagen) was performed in a Roche Life Cycler using 100 ng cDNA. Gene expression calculation was based on the standard curve. Relative expression was defined as sample mRNA expression divided by internal control. Standard curves for the internal control gene cyclophilin (peptidylprolyl isomerase A, PPIA) or CKβBP2/CRIF1 were prepared in a RT-PCR assay using serial dilutions of the cDNA. Primers for CKβBP2/CRIF1 were 5′-GGACCCCGCACAGCCAAGATGG-3′ and 5′-GAGGCTGCTGGGTCTTGAGC-3′, for PPIA 5′-GGGCCGCGTCTCCTTTGAGC-3′ and 5′-CATTCCTGGACCCAAAGCGCTC-3′.

### 2.12 Statistical Analysis

Analyses were performed using Student's t-test and Microsoft Excel Tools. Error bars represent ± SEM. Differences were considered significant at $p<0.05$.

## 3. Results

### 3.1 CKβBP2/CRIF1 interacts with ligand-activated AR in yeast

Yeast two hybrid screening of a HeLa cell cDNA library was performed using as bait human AR-481-919 carboxyl-terminal fragment that includes a portion of the N-terminal domain and DNA and ligand binding domains. Approximately $6 \times 10^5$ yeast library colonies were screened and 6 colonies were positive in the βgalactosidase assay. One clone was protein kinase CK2βbinding protein 2 (CKβBP2) (Ahn et al., 2001; Chung et al., 2003) known also as a GADD45γ interacting protein, CR6-interacting factor 1 (CRIF1) and referred to here as CKβBP2/CRIF1.

The interaction of CKβBP2/CRIF1 with AR-481-919 was analyzed using a yeast liquid β galactosidase assay. Yeast strain Y190 transformed with pBDGAL-CAM-AR-481-919 and pGADGH-CKβBP2/CRIF1 or pGADGH empty vector control were grown in selective medium with or without androgen. There was a slight increase in βgalactosidase activity over the control background in the absence of CKβBP2/CRIF1, however activity increased 8-fold over background in the presence of DHT when both CKβBP2/CRIF1 and AR were present (Fig. S1A). This was 4-fold greater than the increase βgalactosidase activity in the absence of CKβBP2/CRIF1, indicating a DHT-dependent interaction of CKβBP2/CRIF1 and AR. The HeLa cell cDNA clone lacks the first 6 amino acid residues MAASVR compared to the published sequence of CRIF1 (Ahn et al., 2001) and there is a single nucleotide (C to T) difference from the published sequence changing Arg 187 to Cys (Fig. S1B).

### 3.2 CKβBP2/CRIF1 over-expressed in COS cells interacts with AR in the nucleus and inhibits AR transcriptional activation

Immunostaining for AR and CKβBP2/CRIF1 over-expressed in COS cells showed nuclear localization in the presence of DHT (Fig. 1A). An androgen-dependent interaction between CKβBP2/CRIF1 and AR was suggested by co-immunoprecipitation in COS cells (Fig. 1B). Immunoprecipitation with FLAG antibody showed AR bound to FLAG-CKβBP2/CRIF1-7-222 and to FLAG-CKβBP2/CRIF1-1-222 in the presence of DHT, with no detectable interaction in the absence of DHT. The results indicate that CKβBP2/CRIF1 binds to the DHT-activated AR.

Chromatin immunoprecipitation showed that following exposure to R1881, AR and CKβBP2/CRIF1 located to chromatin at 3 and 6 h in the region of the PSA gene upstream enhancer (Fig. 1C). AR bound the PSA gene upstream enhancer DNA while CKβBP2/

CRIF1 did not (Fig. 1D), suggesting CKβBP2/CRIF1 was bound to AR at this chromatin site.

### 3.3 Repressor effect of CKβBP2/CRIF1 on DHT-dependent AR transcriptional activity

CV1 cells were transfected with MMTV-Luc and full-length AR together with pSGFLAG-CKβBP2/CRIF1 or pSGFLAG vector control (Fig. 2). Luciferase activity with pSGFLAG-CKβBP2/CRIF1 (0.1 or 0.5 μg) was decreased compared to the pSGFLAG empty vector control. The results suggested a repressor effect of CKβBP2/CRIF1 on AR transactivation. In similar assays, CKβBP2/CRIF1 inhibited transcriptional activity of the human progesterone receptor-B but not the glucocorticoid receptor (Fig. S2).

### 3.4 CKβBP2/CRIF1 inhibits the AR N/C interaction and binding of the p160 coactivator TIF2 to the AR C-terminal region

We used a mammalian two-hybrid assay to test the influence of CKβBP2/CRIF1 on AR N-terminal region binding to the AR C-terminal region (AR N/C interaction) (He et al., 2000, 2001, 2004). HeLa cells were transfected with a 5XGal4Luc3 reporter gene together with GAL-AR-624-919 (AR C-terminal region linked to the GAL4 DNA binding domain) and VP-AR-1-660 (AR N-terminal and DNA binding domain linked to the VP16 activation domain). A strong DHT-dependent AR N/C interaction between VP-AR-1-660 and GAL-AR-624-919 was inhibited by CKβBP2/CRIF1 compared to the pSGFLAG control (Fig. 3A, left).

A similar experiment indicated that CKβBP2/CRIF1 inhibited binding of the p160 coactivator TIF2 to the AR C-terminal region. With co-transfection of control pSGFLAG, VP-TIF2.1 interacted strongly with the C-terminal Gal-AR-624-919, but this interaction was reduced markedly in the presence of CKβBP2/CRIF1 (Fig. 3A, right). Thus, in addition to its inhibitory effect on the AR N/C interaction, CKβBP2/CRIF1 interfered with the binding of TIF2 to the AR C-terminal region.

CKβBP2/CRIF1 inhibition of AR transcriptional activation was overcome by an increase in TIF2 (Fig. 3B). CV1 cells were cotransfected with pSG-AR and the MMTV-Luc reporter vector together with 0.5 μg pSGFLAG control or pSG-CKβBP2/CRIF1 expression vector, each with either pSGTIF2 (0.1 or 0.5 μg) or the same amount of pSGFLAG empty vector control. In the presence of control, TIF2 increased DHT-dependent luciferase activity (Fig. 3B, left). When cells were cotransfected with CKβBP2/CRIF1 (0.5 μg) the increase with TIF2 (0.1 μg) was inhibited. However, the increase with TIF2 (0.5 μg) was not inhibited by CKβBP2/CRIF1 (0.5 μg) (Fig. 3B, right) consistent with competitive inhibition.

### 3.5 CKβBP2/CRIF1 interacts preferentially with the AR C-terminal region in cell-free binding assays

Binding of [$^{35}$S] CKβBP2/CRIF1-7-222 to GST-AR N-terminal, DNA binding domain or C-terminal ligand binding domain was compared in a glutathione-Sepharose affinity matrix assay. CKβBP2/CRIF1 binding to the AR ligand binding domain (AR amino acid residues 624-919) was evident (Fig. 3C), whereas binding to the N-terminal (1-503) and DNA binding domain (544-634) regions of AR was less and revealed only by longer exposure of the x-ray film (not shown). In a similar assay, there was no direct interaction between CKβBP2/CRIF1 and TIF2 (data not shown).

Binding of CKβBP2/CRIF1 to the C-terminal region of AR and the ability of TIF2 to overcome CKβBP2/CRIF1 inhibition of AR transcriptional activity suggested the CKβBP2/CRIF1 and TIF2 interaction sites in the AR C-terminal region overlap. It is known that an L*XX*LL motif in TIF2 interacts with AR activation function 2 (AF2) in the AR C-terminal

region and that TIF2 can compete with the AR N-terminal F*XX*LF motif binding to AF2 (He et al., 1999, 2001, 2004).

### 3.6 CKβBP2/CRIF1 inhibits constitutive transcriptional activity of AR N-terminal-DNA binding domain

An influence of CKβBP2/CRIF1 on the AR N-terminal activation function was explored using the constitutively active AR-1-660 that includes the AR N-terminal region and DNA binding domain. When CKβBP2/CRIF1 was co-transfected in CV1 cells with pCMV-AR-1-660 and MMTV-Luc, AR-1-660 induced activity was inhibited compared to the pSG-FLAG control vector (Fig. 4A).

Melanoma antigen-A11 (MAGE-A11) is an AR coregulator that binds the AR N-terminal F*XX*LF motif region and increases AR transcriptional activity by stabilizing AR and recruiting TIF2 and p300 (Bai et al., 2005, 2008; Askew et al., 2009, 2010). To further characterize the influence of CKβBP2/CRIF1 on the AR N-terminal activation function, CV1 cells were transfected with pCMV-AR-1-660 and PSA-Enh-Luc with and without pSG-MAGE (Fig. 4B). AR-1-660 induced luciferase activity was increased by MAGE-A11 and inhibited by CKβBP2/CRIF1. The results were consistent with an interaction of CKβBP2/CRIF1 with the AR N-terminal region. CKβBP2/CRIF1 may interfere with binding of MAGE-A11, TIF2 or p300 to the AR N-terminal region.

### 3.7 C-terminal region of CKβBP2/CRIF1 is sufficient for inhibition of AR transcriptional activation

The AR inhibitory region of CKβBP2/CRIF1 was investigated using peptide of CKβBP2/CRIF1 (Fig. 5). CKβBP2/CRIF1-158-206 inhibition of DHT-dependent AR transcriptional activity was stronger than CKβBP2/CRIF1-7-157 and similar to that of the nearly full-length CKβBP2/CRIF1-7-222 (Fig. 5A). Since CKβBP2/CRIF1-158-206 contains a nuclear translocation-like sequence, an explanation for its stronger inhibitory activity might be greater availability to AR in nuclei. However, CKβBP2/CRIF1 fragments 7-157 and 158-206 were nuclear in COS cell expression experiments (results not shown) and they co-immunoprecipitated with full-length AR in a DHT-dependent manner (Fig. 5B). The CKβBP2/CRIF1-158-206 peptide was identified by separation on a 4-20% SDS-glycine gradient gel (Fig. S4).

CKβBP2/CRIF1-158–206 contains a predicted αhelical motif sequence [175]FQELL[179]similar to the AR N-terminal F*XX*LF motif that binds AR AF2 (He et al., 2004, 2000). Mutation of the FXXLF motif to [175]FQEAA[179] [i] in the AR N-terminus abolished its ability to bind AR AF2 (He et al. 2000). However, CKβBP2/CRIF1-7–222 containing the mutant sequence [175]FQEAA[179] (F*XX*AA) retained the ability to inhibit AR transcriptional activity (Fig. 5A) and to bind AR in a DHT-dependent manner (Fig. 5C), suggesting the[175]FQELL[179] motif was not required for the inhibitory interaction of CKβBP2/CRIF1-7-222 with AR.

### 3.8 siRNA knockdown of endogenous CKβBP2/CRIF1 in CWR-R1 human prostate cancer cells increases AR transcriptional activity

CWR-R1 cells express AR at levels sufficient for reporter gene analysis of DHT-dependent transcription. CKβBP2/CRIF1 inhibited endogenous AR activity in transient transfection assays in CWR-R1 cells transfected with MMTV-Luc together with pSGFLAG-CKβBP2/CRIF1-2–222 or -7–222 (Fig. 6A).

CWR-R1 cells expressed CKβBP2/CRIF1 protein by immunostaining (not shown). siRNA knockdown indicated a repressor influence of endogenous CKβBP2/CRIF1 on AR

transcriptional activity. In initial experiments, CKβBP2/CRIF1 siRNAs were tested in COS cells for their ability to inhibit overexpression of recombinant CKβBP2/CRIF1. siRNA1 eliminated detectable CKβBP2/CRIF1 protein, whereas siRNA2 reduced it by only about 20% of the control (Fig. 6B). The siRNAs were also tested in DHT-dependent transcription assays using CV-1 cells cotransfected with AR and MMTV-Luc with and without siRNA. In this assay, CKβBP2/CRIF1 inhibition of DHT and AR-stimulated luciferase activity was prevented by siRNA1 but not by siRNA2 (Fig. 6C).

Knockdown of endogenous CKβBP2/CRIF1 in CWR-R1 cells transfected with MMTV-Luc was performed with increasing amounts of siRNA-1 or 2 in the presence and absence of DHT. At each siRNA concentration, DHT-stimulated luciferase activity was 2–3 fold higher with siRNA-1 compared to siRNA-2 (Fig. 6D). The results are consistent with a repressor effect of endogenous CKβBP2/CRIF1 on AR transcriptional activity in CWR-R1 cells. Repression of AR by endogenous CKβBP2/CRIF1 was released by siRNA1 more than by siRNA2.

### 3.9 CKβBP2/CRIF1 mRNA expression in human prostate cancer

AR is expressed in stroma and epithelium of benign prostatic hyperplasia (BPH) and prostate cancer. Levels of CKβBP2/CRIF1 mRNA did not differ significantly in clinical specimens of BPH, androgen-dependent and castration-recurrent prostate cancer tissue (Fig. 7).

### 3.10 CKβBP2/CRIF1 protein is expressed together with STAT3 in stroma and epithelium of prostate cancer

Because of the reported interaction and transcriptional coactivator effect of CKβBP2/CRIF1 on STAT3 (Kwon et al., 2008), we confirmed the direct interaction of CKβBP2/CRIF1 and STAT3 (Fig. S3) and performed immunostaining of CKβBP2/CRIF1 and STAT3 in adjacent microtome thin sections of a prostate cancer tissue microarray to determine whether the two proteins are localized in the same cells. Proteins were analyzed by immunostaining 25 cores each of human BPH, androgen-dependent and castration-recurrent prostate cancer (Fig. 8). Staining of CKβBP2/CRIF1 in BPH was mainly in stromal cells with weaker staining of epithelial cells. CKβBP2/CRIF1 staining was strongest in fibromuscular cells of stroma. In androgen-dependent prostate cancer, CKβBP2/CRIF1 staining remained similar in stroma but was increased in epithelial cells compared to BPH and was mainly in the cytoplasm of epithelial cells (Fig. 8). In castration-recurrent prostate cancer, CKβBP2/CRIF1 staining of epithelium decreased compared to androgen-dependent prostate cancer (Fig. 8). In a subset of cores (20%) CKβBP2/CRIF1 staining was not detected in epithelial cells of castration-recurrent prostate cancer. Boundaries between epithelial and stromal cells were often obscure in castration recurrent prostate cancer.

STAT3 immunostaining was nuclear and cytoplasmic throughout the glandular epithelium and stroma. STAT3 protein was present wherever CKβBP2/CRIF1 protein was expressed in BPH and prostate cancer (Fig. 8). STAT3 is activated in prostate cancer (Dhir et al., 2002), can bind AR (Matsuda et al., 2001) and stimulates the growth of prostate cancer cells (Ni et al., 2000).

## 4. Discussion

CKβBP2/CRIF1 functioned as an AR corepressor by binding the AR C-terminal region and inhibiting the AR N/C interaction, wherein the F*XX*LF αhelical motif ([23]FQNLF[27]) in the AR N-terminus binds the hydrophobic AF2 region in the C-terminus. CKβBP2/CRIF1 binding to the C-terminal region of AR also competed for binding of the p160 coactivator

TIF2 to the AR C-terminal region, an interaction involving a TIF2 αhelical L*XX*LL motif (He et al., 2001, 2004). CKβBP2/CRIF1 did not bind TIF2, but inhibited TIF2 binding to AR. However, higher amounts of TIF2 could overcome CKβBP2/CRIF1 inhibition of AR transcriptional activity in CV1 cells.

Our evidence that CKβBP2/CRIF1 interacts directly with the AR C-terminus differs from another study (Suh JH et al., 2008) where interaction with the AR N-terminus in a pull-down assay was stronger than with the AR C-terminus. The difference may be due to the androgen-dependent nature of CKβBP2/CRIF1 binding to the AR ligand-binding domain. However, we cannot rule out that the hinge region 33 amino acids 624-656, present in our construct but not that of Shu J.H. et al. 2008, played a role in the binding difference with Shu J.H. et al.

We found that CKβBP2/CRIF1 also inhibited the constitutive transcriptional activity of AR-1–660, an AR N-terminal and DNA binding domain fragment, alone or in the presence of MAGE-A11. This inhibition of transcriptional activity may reflect an interaction of CKβBP2/CRIF1 with the AR N-terminal region, although this appeared weaker than the androgen-dependent binding of CKβBP2/CRIF1 to the AR C-terminal domain in affinity matrix assays.

MAGE-A11 is an AR coregulator that interacts with the AR N-terminal F*XX*LF motif region. Binding of MAGE-A11 to the F*XX*LF motif can compete with the AR N/C interaction between the AR F*XX*LF motif and AF2 in the AR ligand binding domain (Bai et al., 2005; Bai and Wilson, 2008). MAGE-A11 increases AR transcriptional activity by binding and promoting AR complexes with TIF2 (Askew et al., 2009) and p300 (Askew et al., 2010). p160 coactivators such as TIF2 bind the AR N-terminal region (Bevan et al., 1999). However, an intermediary protein may modulate these p160 interactions with the unstructured AR N-terminus (Lavery and McEwan, 2008a,b). In prostate cancer cells with higher MAGE-A11 protein expression, TIF2 may be linked to the AR N-terminus in part through its binding to MAGE-A11 (Askew et al., 2009). This suggested that CKβBP2/CRIF1 might also inhibit AR action by interfering with coregulators that bind the AR N-terminal region.

The AR N/C interaction brings together the unstructured N-terminal region with the highly structured C-terminal region and has a stabilizing effect on AR by slowing the dissociation rate of bound DHT (He et al., 2002, 2004, 2006). The AR N/C interaction may influence coactivator interacting surfaces in AF1 in the N-terminal region (Lavery and McEwan, 2008a,b). Several lines of evidence indicate that the AR N/C interaction has an essential role in transcriptional activity (Li et al., 2006). AR mutations that interfere with the N/C interaction or its effects on AR coregulator interactions are associated with partial androgen insensitivity in humans (Quigley et al., 2004; Lagarde et al., 2012). Moreover, inhibition of the AR N/C interaction by other AR corepressors (Burd et al., 2006) may reflect the importance of this domain interaction in AR functional activity.

Other AR corepressors that inhibit the AR N/C interaction are cyclin D1 (Burd et al., 2005, 2006), SMRT (Dotzlaw et al., 2002; Liao et al., 2003) and NCoR (Cheng et al., 2002; Berrevoets et al., 2004; Wu et al., 2006; Hodgson et al., 2008) that interacts with both N and C terminal regions of AR. NCoR inhibition of AR resembles that of CKβBP2/CRIF1 in that its AR inhibitory effect in the presence of R1881 can be reversed by TIF2 (Berrevoets et al., 2004), suggesting competition between NCoR and TIF2 for binding sites on ligand activated AR. Binding of NCoR to AR can be increased by antiandrogens (Dotzlaw et al., 2002; Berrevoets et al., 2004; Wu et al., 2006; Hodgson et al., 2008).

In the PPC-1 cell line derived from a primary human prostate cancer, the histone deacetylase inhibitor trichostatin A abolished the repressor activity of CKβBP2/CRIF1 on AR transcriptional activity and this effect was dependent on histone deacetylase-4 (HDAC-4) activity (Suh et al., 2008). HDAC-4 was present in nuclei of castration-recurrent prostate cancer (Halkidou et al., 2004). Class 1 HDACs 1–3 are also expressed in prostate cancers (Witt et al., 2009). We found a variety of HDAC mRNAs in a small group of prostate cancers including HDAC-4 (data not shown). A number of AR corepressors recruit HDACs (Burd et al., 2006; Gross et al., 2004) and may form complexes that control HDAC turnover (Guo et al., 2012).

The AR inhibitory activity of CKβBP2/CRIF1 in CV1 cells resided mainly in its C-terminal region. Similarly, C-terminal CKβBP2/CRIF1-99–222 exhibited stronger AR binding than the N-terminal region in a yeast interaction assay and had stronger AR repressor activity in HEK-293 cells (Suh et al., 2008). We identified a 49 amino acid CKβBP2/CRIF1-158–206 fragment that retained AR binding activity and strong inhibition of AR transcriptional activity. This fragment contains a potential coiled-coil region of α-helices and includes an F*XX*LL motif similar to the AR N-terminal F*XX*LF motif, suggesting that like the AR FXXLF motif, it might confer binding to AR AF2. However, mutation of this motif to F*XX*AA in CKβBP2/CRIF1 7-222 did not alter binding to full-length AR and caused only slight inhibition of AR transcriptional activity compared to the parent fragment containing F*XX*LL. In murine C2C12 skeletal muscle cells, C-terminal CKβBP2/CRIF1-99-222 was a stronger inhibitor of Nurr-77 transactivation than was the N-terminal region of CKβBP2/CRIF1 (Park et al., 2005).

CKβBP2/CRIF1 was reported to enhance the actions of GADD45 (Chung et al., 2003), a p53 induced DNA damage and cell stress response protein (Carrier et al., 1999; Liebermann and Hoffman, 2008). As participants in the cell response to DNA damage, CKβBP2/CRIF1 and other corepressors, forkhead transcription factor FoxO1 (Ma et al., 2009) and checkpoint protein hRAD9 (Wang et al., 2004), also inhibited the AR N/C interaction. FoxO1 binding to the AR N-terminal region interfered with the AR N/C interaction, blocked N-terminal binding of a p160 coactivator and inhibited AR transcriptional activation. AKT phosphorylation of FoxO1 prevented its AR corepressor activity and PTEN suppressed this action of AKT. FoxO1 repressor activity was also inhibited by CDK2 activity, and when CDK2 activity was abolished in response to DNA damage, FoxO1 was activated (Huang and Tindall, 2007). In this connection, CKβBP2/CRIF1 was also reported to inhibit CDK2 activity (Chung et al., 2003) and thereby may contribute to an increase in FoxO1 corepressor activity. hRAD9 is a member of the RAD family of checkpoint proteins and a multifunctional sensor and regulator of the cellular response to DNA damage involved in DNA repair, checkpoint activation, cell cycle regulation and apoptosis signaling (Ishikawa et al., 2007). An F*XX*LF motif in the hRAD9 C-terminal region interacted with the AR C-terminal region, presumably AF2, inhibited the AR N/C interaction and AR transcriptional activation (Wang et al., 2004).

Loss of CKβBP2/CRIF1 may have a growth stimulating effect in some cells as a consequence of removing growth inhibition by CKβBP2/CRIF1 unrelated to AR. For example, NAC-1, a BTB/POZ protein, repressed transcription of the CKβBP2/CRIF1 gene. In HeLa cells, the increase in CKβBP2/CRIF1 expression resulting from silencing NAC-1 was associated with growth inhibition in cell culture and xenographs (Nakayama et al., 2006). Since HeLa cells contain low levels of AR and their growth is not stimulated by androgen, inhibition of growth associated with an increase in CKβBP2/CRIF1 protein may have resulted from tumor suppressor effects of CKβBP2/CRIF1 similar to those of GADD45y Similarly, castration recurrent prostate cancer epithelial cells with a lower

amount of CKβBP2/CRIF1 protein may have a selective growth advantage due to increased transcriptional activity of AR.

Recent studies suggest that CRIF1 has a role in oxidative phosphorylation through the integration of polypeptides into the mitochondrial membrane (Kim et al., 2012l; Shin et al. 2013) and that is part of the mammalian ribosome (Koc et al. 2013). In addition, CRIF1 has a role in oxidative phosphorylation in mouse adipose tissue (Ryu et al. 2013). Other evidence supports multiple functions of CKβBP2/CRIF1 in transcription factor regulation. CKβBP2/CRIF1 is a coactivator of ELF3 (E74-like factor 3) (Kwon et al., 2009) and STAT3 (Kwon et al., 2008). CKβBP2/CRIF1 down-regulates the transcription factor NRF2 by promoting ubiquitination and degradation (Kang et al., 2010). In our studies, CKβBP2/CRIF1 was an activator of the cytomegalovirus promoter but not the simian virus 40 promoter (Fig. S5).

Multifunctional effects of CKβBP2/CRIF1 may regulate actions of AR, STAT3 and other transcription factors in prostate reactive stroma and in epithelium that control vascularization and growth of prostate cancer (Berry et al., 2008; Yang et al., 2005; Montecinos et al., 2012).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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**Highlights**

- CKβBP2/CRIF1 is a multifunctional corepressor of androgen receptor (AR) transcriptional activity through inhibition of the AR N/C interaction

  CKβBP2/CRIF1 binds STAT3 and STAT3 is expressed with CKβBP2/CRIF1 in prostate cancer

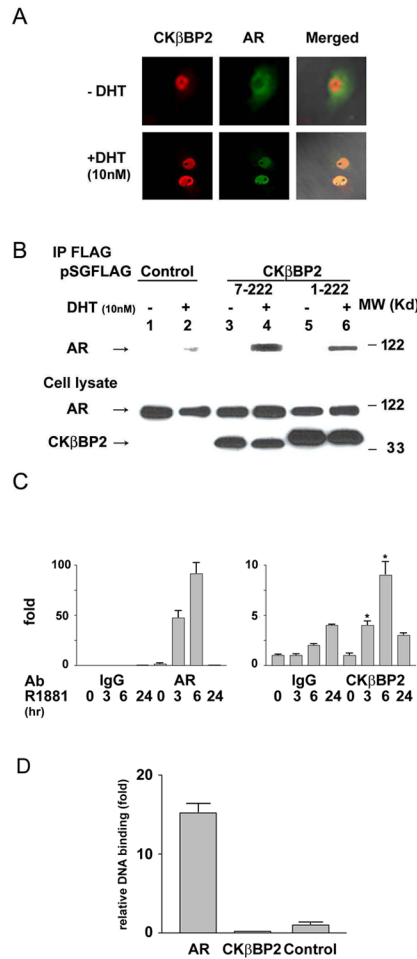  action of STAT3 on prostate cancer may offset CKβBP2/CRIF1 inhibition of AR and allow prostate growth

**Figure 1. CKβBP2/CRIF1 interacts with AR and colocalizes with AR in chromatin**
(**A**) CKβBP2/CRIF1 colocalized with AR in nuclei. pSGFLAG-CKβBP2/CRIF1 and pSG-AR were cotransfected into COS cells in the absence (top) or presence (bottom) of 10 nM DHT. Immunofluoresence for AR (green) and CKβBP2/CRIF1 (red) shows CKβBP2/CRIF1 shows typical nuclear and perinuclear localization in the absence of DHT and nuclear in the presence of DHT while AR is cytoplasmic in the absence of DHT and nuclear in the presence of DHT. Nuclear colocalization of CKβBP2/CRIF1 and AR in the presence of DHT is demonstrated by merged images (yellow). (**B**) FLAG-CKβBP2/CRIF1 coimmunoprecipitated with AR in the presence of DHT. AR was cotransfected in COS cells with control pSGFLAG (lanes 1, 2), truncated FLAG-CKβBP2/CRIF1 (aa 7–222, lanes 3, 4) or full-length FLAG-CKβBP2/CRIF1 (aa 1–222, lanes 5, 6) in the absence (-) and presence (+) of 10 nM DHT. Protein extracts were immunoprecipitated with FLAG monoclonal antibody affinity resin and AR (indicated by arrow) was visualized by western blotting with AR specific antibody AR32 (30). CKβBP2/CRIF1 in cell extracts was analyzed by western blotting using FLAG monoclonal antibody. (**C**) Chromatin immunoprecipitation was performed in CWR-R1 cells showing endogenous AR and CKβBP2/CRIF1 localization at the PSA gene enhancer at 3 and 6 hrs compared to IgG control *p<0.01. (**D**) DNA binding was assayed in COS cells as described in *Experimental Procedures*. In contrast to AR, CKβBP2/CRIF1 did not bind to PSA enhancer DNA.
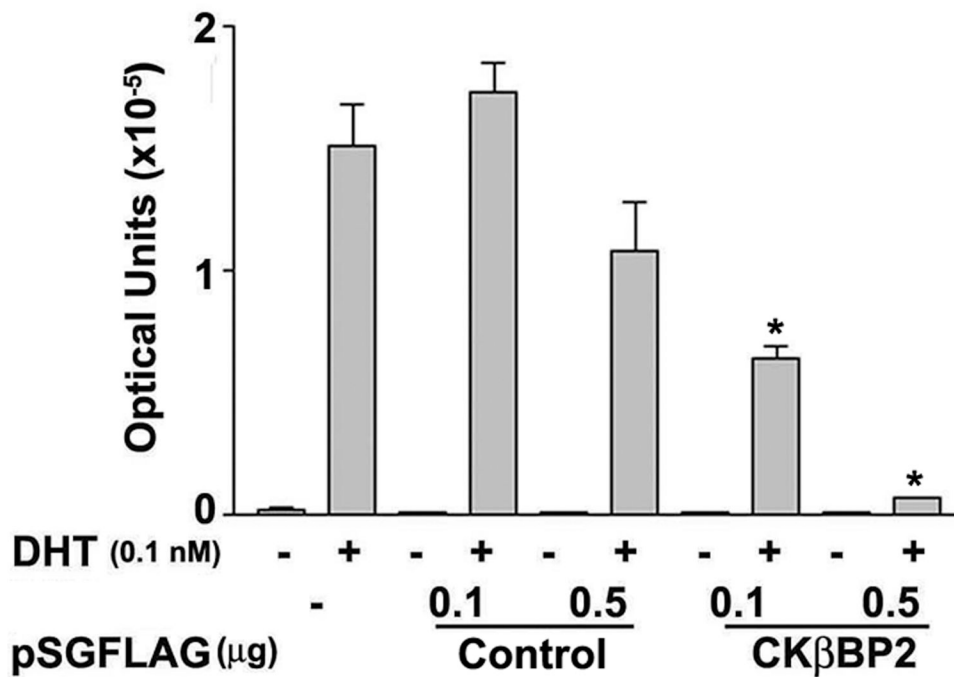
**Figure 2. CKβBP2/CRIF1 inhibits AR transcriptional activation of MMTV-Luc in transient cotransfection assays**
CV1 cells in 6 cm dishes were cotransfected with MMTV-Luc (0.5 µg) and pSG-AR (0.1 µg) together with pSG5FLAG control (0.1 and 0.5 µg) or pSGFLAG-CKβBP2/CRIF1-7-222 (0.1 and 0.5 µg). Cells were incubated with and without 0.1 nM DHT and luciferase activity assayed. Error bars represent ± SEM. CKβBP2/CRIF1 repressed AR transactivation at 0.1 µg *(p<0.001), and 0.5 µg *(p<0.001) compared to pSG5FLAG control 0.01 µg and 0.5 µg respectively.
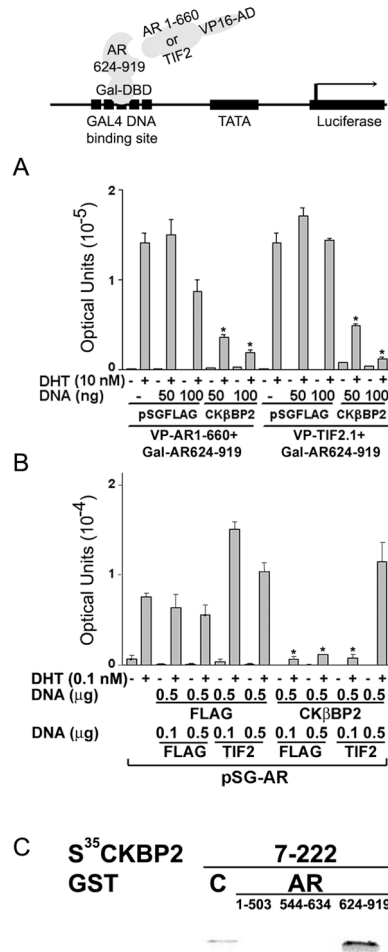
**Figure 3. CKβBP2/CRIF1 inhibits AR N-terminal and TIF2 interactions with the AR C-terminal region**

Schematic of mammalian two-hybrid assay is shown at the top. (**A**) Gal-AR-624–919 (AR C-terminal region) (50 ng) and 0.1 μg 5XGAL4Luc were cotransfected into HeLa cells with 50 ng VP-AR-1-660 (AR N-terminal region and DNA binding domain) or 50 ng VP-TIF2.1 (TIF2-624-1287) together with pSGFLAG control or pSGFLAG CKβBP2/CRIF1 expression vectors. Cells were incubated with and without 10 nM DHT and luciferase activity was measured. CKβBP2/CRIF1 inhibited both AR1-660 and TIF2 interactions with AR 624-919 ** (p<0.01) compared to equal amount of pSGFLAG control. (**B**) CKβBP2/CRIF1 inhibition of AR transcriptional activation rescued by TIF2. CV1 cells in 6 cm dishes were co-transfected with pSG-AR (0.1 μg) and MMTV-Luc (0.5 μg) together with pSGFLAG-CKβBP2/CRIF1 (0.5 μg) or pSGFLAG control (0.5 μg), each with pSGFLAG-TIF2 expression vector (0.1 μg or 0.5 μg) or the same amounts of control vector. Cells were incubated in the absence and presence of 0.1 nM DHT and luciferase activity assayed. CKβBP2/CRIF1 repressed AR transactivation in the presence of pSGFLAG control and TIF2 0.1 μg *(p<0.01) but not in the presence of TIF2 0.5 μg (**C**) Preference for binding of CKβBP2/CRIF1 to the AR ligand binding domain. Glutathione-Sepharose affinity matrix assay of [$^{35}$S]CKβBP2/CRIF1-7-222 binding to GST-glutathione-Sepharose control (C), GST-AR-1-503 N-terminal domain, GST-AR-544-634 DNA binding domain, GST-AR-624-919 ligand binding domain. Binding was assayed in the presence of 0.1 nM DHT. Error bars represent ± SEM.
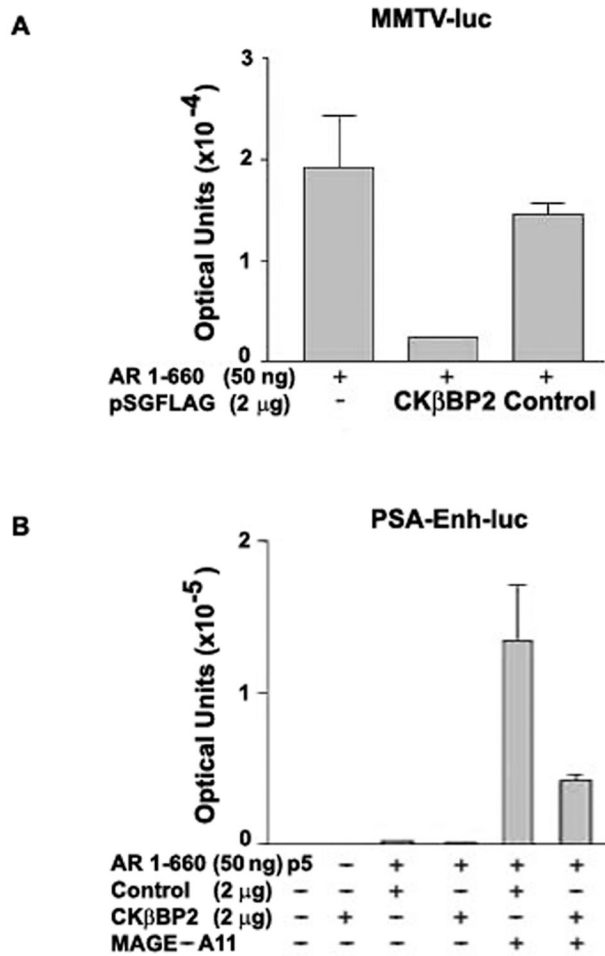
**Figure 4. Constitutive transcriptional activity of AR-1-660 is inhibited by CKβBP2/CRIF1 in the absence and presence of MAGE-A11 or MAGE-A11 plus the p160 coactivator, TIF2**
(**A**) CV1 cells in 6 cm dishes were transfected with pCMVAR-1-660 (50 ng) and MMTV-Luc (5 μg) together with pSG-FLAG-CKβBP2/CRIF1 or pSG-FLAG-vector control (2 μg). Cells were incubated without DHT and luciferase activity assayed. (**B**) CV1 cells were transfected with pCMVAR-1-660 (50 ng) and PSA-Enh-Luc (5 μg) together with pSG-FLAG-CKβBP2/CRIF1 or pSG-FLAG-vector control (2 μg), each in the presence or absence of the AR coregulator pSG-MAGE-A11 (2 μg). p5 indicates pCMV control vector for AR. Cells were incubated in the absence of hormone and luciferase activity assayed. Error bars represent ± SEM. Results are representative of three experiments, each done in triplicate.
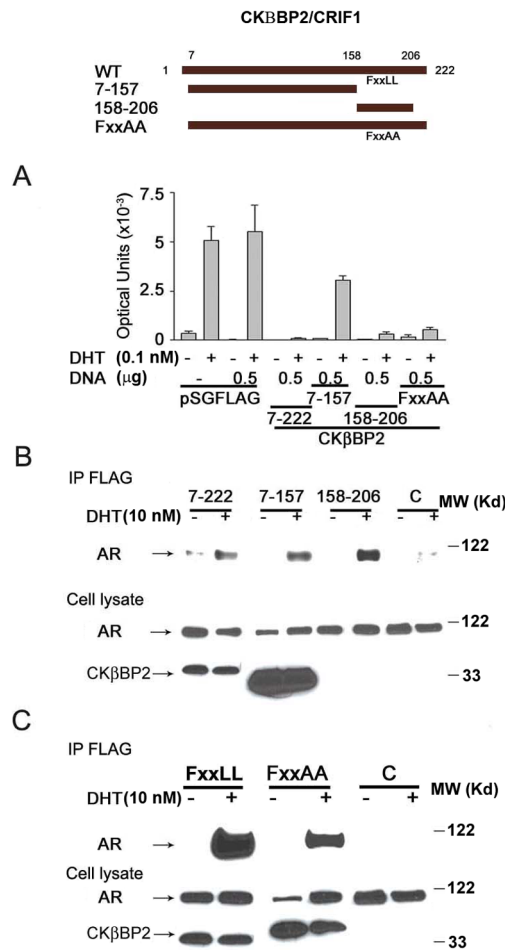
**Figure 5. Regional sites of CKβBP2/CRIF1 and AR interactions**

Schematic of CKβBP2/CRIF1 full-length (amino acid residues 1-222) is shown with CKβBP2/CRIF1 fragments 7-157, 158-206 and 7-222 containing a mutation of the F*XX*AA motif. (**A**) C-terminal region of CKβBP2/CRIF1-158-206 was sufficient for AR transcriptional repression. MMTV-Luc (0.5 µg) and pSGFLAG-AR (0.1 µg) were cotransfected in CV1 cells in 6 cm dishes with and without control vector pSGFLAG (0.5 µg), CKβBP2/CRIF1-7-222, 7-157, 158-206 or 7-222 with F*XX*LL ([175]FQELL[179]) mutated to F*XX*AA. Incubations were in the presence or absence of 0.1 nM DHT. Error bars represent ± SEM. (**B**) CKβBP2/CRIF1 N-terminal residue 7-157 and C-terminal residue 158-206 fragments interact with full-length AR. AR interaction with FLAG-CKβBP2/CRIF1 amino acid residues 7-222, 7-157 and 158-206 and the pSGFLAG empty vector control was compared by coimmunoprecipitation assay in lysates of cotransfected COS cells incubated in the presence and absence of 10 nM DHT. Immunoprecipitation was preformed using FLAG affinity resin antibody and AR was visualized by western blotting using AR 32 antibody. Expression levels of AR and CKβBP2/CRIF1 were assayed by western blotting transfected COS cell protein extracts. FLAG-CKβBP2/CRIF1 158-206 was too small to identify on this gel. Its expression is shown on a 4-20% gradient gel (Fig. S4) (**C**) The F*XX*LL motif of CKβBP2/CRIF1 is not required for AR binding. FLAG-CKβBP2/CRIF1-7-222 wild-type or with [175]FQEAA[179] F*XX*AA mutation and control pSGFLAG empty vector were cotransfected individually with AR into COS cells and incubated in the presence and absence of 10 nM DHT. Immunoprecipitation was performed using FLAG

antibody affinity resin and AR was visualized by western blotting with AR 32 antibody. Expression levels of CKβBP2/CRIF1 and AR in COS cell protein extracts were assayed by western blotting. Error bars represent ± SEM. Results are representative of three experiments.
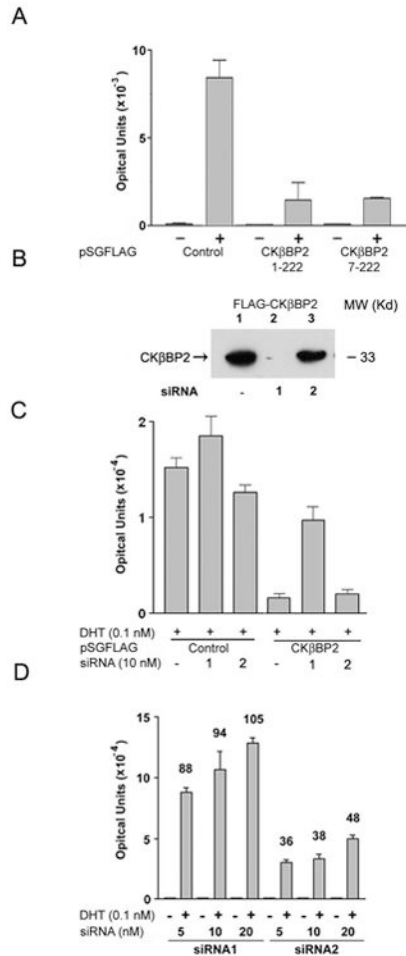
**Figure 6. CKβBP2/CRIF1 inhibits endogenous AR transcriptional activation in CWR-R1 human prostate cancer cells**
(**A**) CWR-R1 cells in 6 well plates were transfected with MMTV-Luc (0.1 µg) and 0.25 µg pSG5FLAG-control vector, pSGFLAG-CKβBP2/CRIF1-1-222 or pSGFLAG-CKβBP2/CRIF1-7-222. Cells were incubated with and without 0.1 nM DHT and luciferase activity measured. Error barsrepresent ± SEM. (**B**) Inhibition of recombinant CKβBP2/CRIF1 protein expression by CKβBP2/CRIF1 siRNA 1. COS cells in 6 cm dishes were transfected with pSGFLAG-CKβBP2/CRIF1 (lane 1), pSGFLAG-CKβBP2/CRIF1 with the addition of 20 nM CKβBP2/CRIF1 siRNA 1 (lane 2) or siRNA 2 (lane 3). Cells were incubated for 48 h and protein levels in cell lysates assayed by western blotting using FLAG monoclonal antibody. (**C**) CKβBP2/CRIF1 siRNA-1 prevented recombinant CKβBP2/CRIF1 inhibition of AR transcriptional activation. CV1 cells in 6 cm dishes were transfected with 0.5 µg MMTV-Luc, 0.1 µg pSG-AR and 0.5 µg pSGFLAG-CKβBP2/CRIF1 or control vectors, with addition of 10 nM CKβBP2/CRIF1 siRNA-1 or siRNA-2. Cells were incubated with 0.01 nM DHT and luciferase activity assayed. Error bars represent ± SEM. (**D**) Endogenous AR transcriptional activation in CWR-R1 cells was increased by inhibition of CKβBP2/CRIF1 expression. CWR-R1 cells in 6 well plates were transfected with MMTV-Luc (0.1 µg) using Effectene and increasing amounts (5-20 nM) CKβBP2/CRIF1 siRNA-1 or siRNA-2. Cells were incubated in the presence and absence of DHT and luciferase activity assayed. Fold inductions indicated by the number above the bar represent luciferase activity in the presence of DHT divided by activity in the absence of DHT. Error bars represent ± SEM. Results are representative of two experiments, each performed in triplicate.
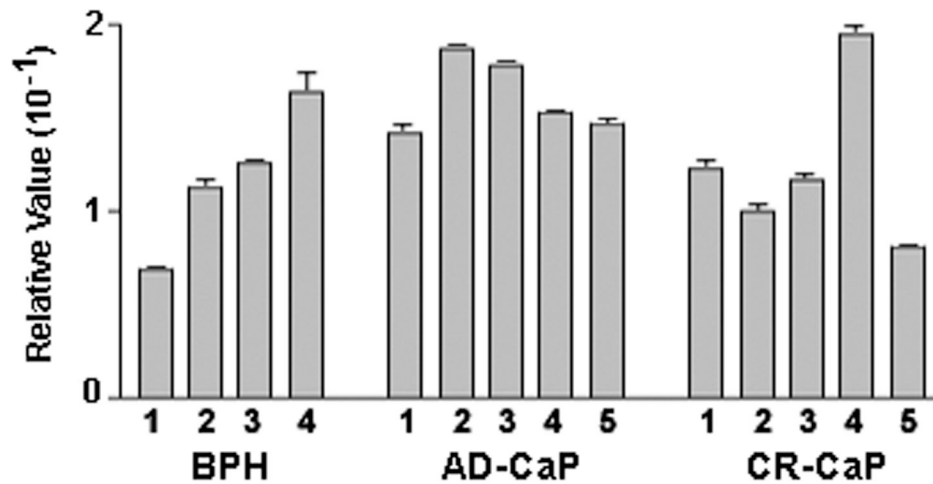
**Fig. 7. CKβBP2/CRIF1 mRNA in benign prostatic hyperplasia (BPH), androgen-dependent prostate cancer (AD-CaP) and castration-recurrent prostate cancer (CR-CaP)**
RNA was extracted using Trizol (Qiagen) from 4 or 5 different specimens of BPH, AD-CaP and CR-CaP and reverse transcribed using Super Script II Reverse Transcriptase (Invitrogen). RT-PCR using 100 ng cDNA was performed in a Roche Light Cycler with Quanti Tech SYBR Green (Qiagen). Gene expression was calculated using a standard curve. Relative expression was defined as sample gene expression divided by internal control gene expression as described in Methods. Error bars represent ± SEM. Results are representative of two experiments, each performed in triplicate.
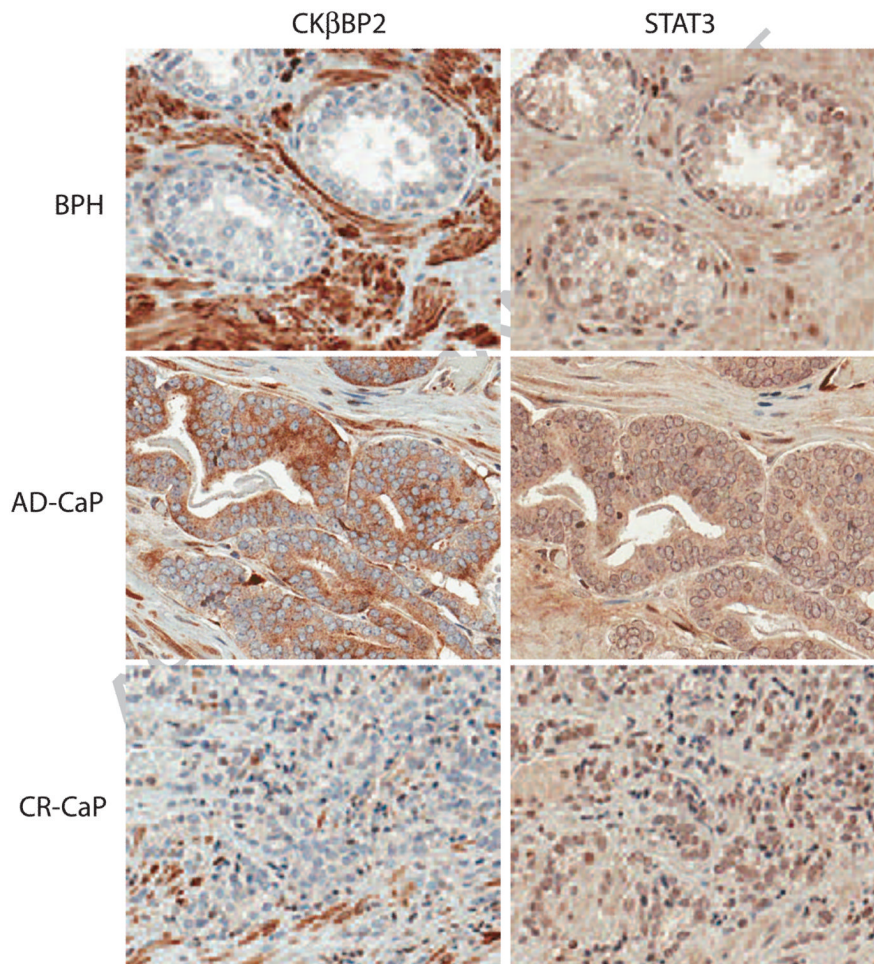
**Figure 8. CKβBP2/CRIF1 and STAT3 expression in adjacent sections of tissue microarrays of human benign prostatic hyperplasia, androgen-dependent and castration-recurrent prostate cancer**

Representative pictures show human benign prostatic hyperplasia (BPH, top), androgen-dependent (AD-CaP, middle) and castration-recurrent prostate cancer (CR-CaP, bottom). Tissue microarray adjacent thin sections were prepared and immunostained as described in Methods. CKβBP2/CRIF1 (CKβBP2, left) and STAT3 (right). CKβBP2/CRIF1 protein in epithelial cells: AD-CaP > BPH (p<0.001), CR-CaP < AD-CaP (p<0.014); CKβBP2/CRIF1 protein in stromal cells: staining is mainly in myofibroblasts. No difference overall between staining of stroma in BPH, AD-CaP and CR-CaP. STAT3 protein was present where CKβBP2/CRIF1 protein was expressed in BPH and prostate cancer.